Biosynthesis of Cruciferous Phytoalexins†

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Abstract: We report the results of biosynthetic studies on the sulfur-containing indole phytoalexins, antimicrobial compounds produced by plants after exposure to microorganisms. Feeding experiments with UV-irradiated sliced turnip root (*Brassica campestris* ssp. *rapa*) revealed that spirobrassinin (**3**) is formed *via* brassinin (**1a**) and that **1a** is biosynthesized by the intramolecular rearrangement of L-tryptophan to form unstable indol-3-ylmethyl isothiocyanate (**10a**). Trapping experiments of **10a** with $NaSCH_3$ and formation of $PhCH_2NH$-CS-SCH_3 (**14**) on administration of benzyl isothiocyanate supported the presence of the labile isothiocyanate intermediate. Feeding experiments of a mixture of L-[*methyl*-$^3H_3$]methionine and L-[^{35}S]methionine revealed that the methylthio group of methionine is incorporated into **1a** as an unusual intact unit. Incorporation of L-[^{35}S]cysteine into **1a** and **3** indicated the origin of another sulfur atom. These studies suggest that the biosynthetic pathway of cruciferous phytoalexins is at least partially linked to one of the indole glucosinolates which are typical components of crucifers. Structures of metabolites of 2-methylbrassinin (**16**) imply the presence of an oxidized intermediate between **1a** and other cruciferous phytoalexins.

Introduction

Plants produce antimicrobial compounds called phytoalexins when they are exposed to microorganisms. Accumulation of phytoalexins around infection sites is considered as one of the important antimicrobial defense mechanisms of the plant. To date, more than 200 phytoalexins have been isolated from more than 20 plant families.[1] However, until our report[2] no phytoalexins had been obtained from the plant family Cruciferae even though members of this family include many economically important vegetables. The first three cruciferous phytoalexins isolated from Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis*) inoculated with the bacterium *Pseudomonas cichorii* are named brassinin (**1a**), methoxybrassinin (**1b**), and cyclobrassinin (**2**) and have structurally unique features that include an indole ring and two sulfur atoms. Additional cruciferous phytoalexins have been isolated from Japanese radish (*Raphanus sativus*, spirobrassinin (**3**)),[3] cabbage (*B. oleracea* L., 4-methoxybrassinin (**1c**), methoxybrassenin A (**6a**) and B (**6b**), brassicanal C (**7b**)),[4] turnip (*B. rapa* or *B. campestris*),[5] rape (*B. napus* L. var. *oleifera* L.),[6] Indian mustard (*B. juncea*, brassilexin (**5**)),[7] false flax (*Camelina sativa*, camalexin (**8a**), methoxycamalexin (**8b**)),[8] arabidopsis (*Arabidopsis thaliana* L. *Heynhold*),[9] and Chinese cabbage (brassitin (**1d**), methoxybrassitin (**1e**), brassicanal A (**7a**)),[10] as shown in Figure 1. All these phytoalexins are structurally related to indole or indole derivatives possessing one or two sulfur atoms.

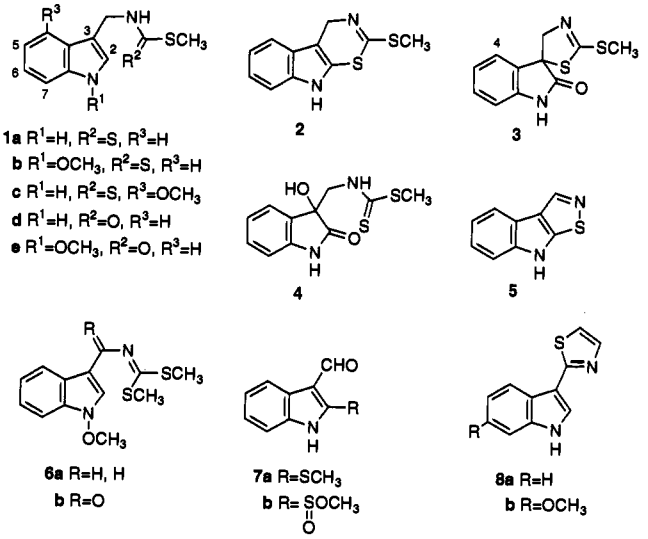

Figure 1. Structures of representative cruciferous phytoalexins: **1a**, brassinin. **1b**, methoxybrassinin; **1c**, 4-methoxybrassinin; **1d**, brassitin; **1e**, methoxybrassitin; **2**, cyclobrassinin; **3**, spirobrassinin; **4**, dioxibrassinin; **5**, brassilexin; **6a**, methoxybrassenin A; **6b**, methoxybrassenin B; **7a**, brassicanal A; **7b**, brassicanal C; **8a**, camalexin; **8b**, methoxycamalexin.

Cruciferous plants contain thioglucosides called glucosinolates, which undergo enzymatic hydrolysis on crushing of the plant tissues to give isothiocyanates, nitriles, alcohols, and so on.[11] Mustard oil, which is a mixture of these decomposition products, has antimicrobial and anti-insect properties. The glucosinolates

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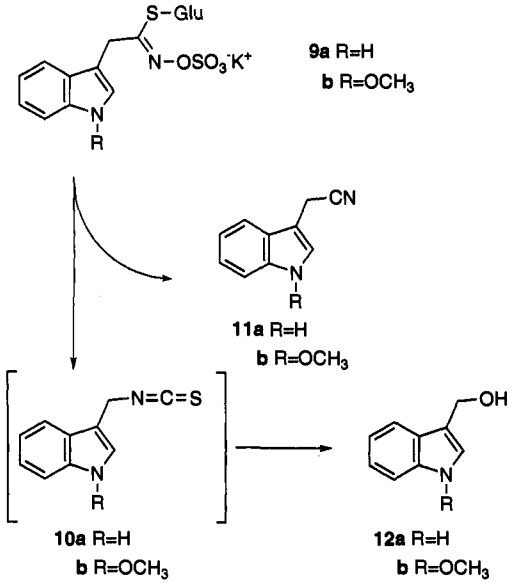
Scheme 1. Structures and Enzymatic Hydrolysis Products of Indole Glucosinolates



are therefore regarded as important defense compounds in crucifers against microorganisms and insects.[12]

Enzymatic hydrolysis converts the indole glucosinolates, neoglucobrassicin and glucobrassicin, to (1-methoxyindol-3-yl)-methyl isothiocyanate (**10b**) and indol-3-ylmethyl isothiocyanate (**10a**), respectively (Scheme 1). Although the latter isothiocyanate, **10a**, is too unstable to isolate,[13] the addition of methanethiol, a known breakdown product in cruciferous plants, to isothiocyanates could formally yield **1b** and **1a**. However, these phytoalexins do not appear to be hydrolysis products of indole glucosinolates, because none of these compounds was detected in the extract of enzymatic hydrolysis products.[2c] Therefore, a different biosynthetic pathway might be involved in the synthesis of these cruciferous phytoalexins. Structural similarities between the two types of compounds suggest that they may share some biosynthetic steps.

In a previous work based on feeding experiments of deuterium- and ^{13}C-labeled compounds,[14] we found that brassinin is biosynthesized from L-tryptophan by a Lossen-type molecular rearrangement and that it is a precursor of both cyclobrassinin and spirobrassinin. Moreover, time course studies[5a] of phytoalexins and glucosinolates in UV-irradiated turnip root tissue (*B. campestris* L. ssp. *rapa*) indicated that the levels of both phytoalexins and indole glucosinolates increased in the UV-irradiated tissue, whereas only the latter increased in nonirradiated control tissue. These facts suggest the possibility that a biosynthetic relationship may exist between the indolic phytoalexins and indole glucosinolates and that induction of additional enzymes would be required for phytoalexin formation.

In the present paper, we describe the results of detailed biosynthetic studies on cruciferous phytoalexins using UV-irradiated turnip root tissue. Evidence for the isothiocyanate intermediate and the intact addition of the methylthio group of L-methionine are described. The results indicated a link between biosynthesis of indole phytoalexins and glucosinolates. Furthermore, a plausible diol or diol related intermediate linking **1a** and other indolic phytoalexins is proposed from the results of a 2-methylbrassinin (**16**) feeding experiment. Structures of two minor phytoalexins isolated from Japanese radish also support the intermediate.

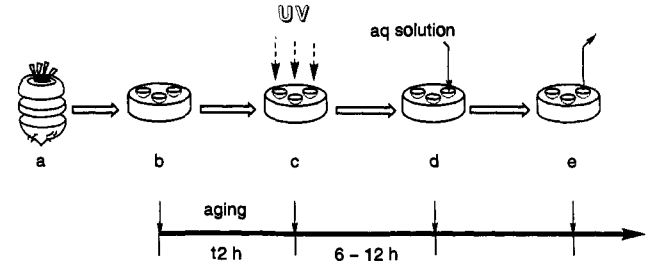


Figure 2. Feeding experiment methodology: (a) cutting turnips horizontally, (b) making hemispherical holes, (c) irradiation with ultraviolet for 10 min, (d) adding labeled compounds as a 0.1% Tween 80 aqueous solution, (e) HPLC analysis and harvesting.

Results

Biosynthetic Correlation of Major Phytoalexins. In view of their structures, a plausible biogenesis of four representative phytoalexins is proposed. Brassinin (**1a**) is oxidatively cyclized to cyclobrassinin (**2**), and the latter is oxidized to dioxibrassinin (**4**), which has been isolated as a minor phytoalexin of *P. cichorii*-inoculated cabbage.[4b] Finally, compound **4** cyclizes to give spirobrassinin (**3**). Chemical transformations of **1a** to **2**[2b] and of **4** with thionyl chloride to **3**[15] have been accomplished.

Time course studies of the phytoalexins in UV-irradiated turnip tissue[5a] have also suggested the same biosynthetic pathway. That is, the compounds **1a** and **1b** were first produced approximately 1 day after UV irradiation, and then **2** was produced with the decline of **1a** after 2 days. Finally, **3** was produced with the decline of **2** and 1 week after the decline of **1a**. These results are consistent with the proposed biogenesis.

To examine biogenesis, three deuterium-labeled phytoalexins (**1a, 2, 4**) were synthesized and several feeding experiments with the labeled compounds were carried out. Brassinin (**1a**) labeled at the methyl group of its side chain was readily synthesized from 3-(aminomethyl)indole[16] by treatment with CS_2 and C^2H_3I. Cyclobrassinin (**2**) labeled at the same position was then synthesized from the labeled **1a**. Deuterium-labeled **4** was synthesized from a corresponding amine[17] by the same procedure. The ^1H-NMR spectra of synthetic [*methyl*-$^2H_3$]brassinin, cyclobrassinin, and dioxibrassinin indicated the deuterium content at the methyl group to be at least 99%.

In a feeding experiment with the labeled brassinin, [*methyl*-$^2H_3$]brassinin (0.88 mM, 800 mL) was administered to the UV-elicited turnip tissue (Figure 2) and the progress of the biosynthesis was monitored using the HPLC method described in a previous paper.[18] The aqueous phase was harvested at an earlier stage (27 h after the administration) to avoid dilution with increasing unlabeled natural **1a**. The ethyl acetate extract from the water phase was separated by silica gel chromatography to give 2 mg of **2** and 11 mg of **3**. The ^1H-NMR spectra of the two compounds indicated effective incorporation of the ^2H label into cyclobrassinin (*methyl*-$^2H_3$, 75%) and spirobrassinin (*methyl*-$^2H_3$, 81%). Incorporation was estimated by the decline in signal area of the methyl group in each ^1H-NMR spectrum. Both **2** and **3** are therefore biosynthesized from **1a**.

To confirm step **2** to **3**, a feeding experiment with [*methyl*-$^2H_3$]cyclobrassinin was conducted. Although the feeding experiments were done under several different conditions, [*methyl*-$^2H_3$]cyclobrassinin was not incorporated into **3**. Moreover,

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(15) Dioxibrassinin (**4**) was converted to spirobrassinin (**3**) in 98% yield by treating with $SOCl_2$.

(16) Schallenberg, J.; Meyer, E. *Z. Naturforsh., B* **1983**, *38*, 108–112.

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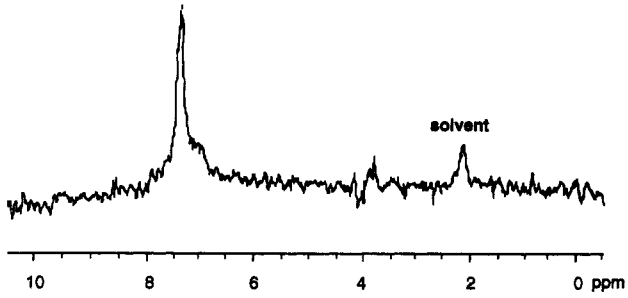


Figure 3. ^{2}H-NMR spectrum (61.44 MHz, 10-mm tube) of spirobrassinin (**3**) derived from L-[4′-^{2}H]tryptophan as a solution in CCl_4—cetone (9:1).

[*methyl*-$^2H_3$]dioxibrassinin, a plausible precursor to **3**, was not incorporated into spirobrassinin.

These results indicated that **1a** is an advanced precursor of **2** and **3** and that **3** is biosynthesized directly from **1a**, not by way of cyclobrassinin or dioxibrassinin. In the feeding experiment with [*methyl*-$^2H_3$]brassinin, the incorporation ratio of spirobrassinin (81%) was higher than that of cyclobrassinin (75%), supporting the hypothesis that spirobrassinin is not biosynthesized *via* cyclobrassinin. In a later section, a different pathway from **1a** to **3** will be discussed.

Interestingly, the deuterium content of **4** recovered from the feeding experiment was 29%, in contrast to the initial content of 99%. This means that a facile exchange of the deuterium label in **4** occurred during the 6-h incubation period. On the other hand, high incorporation of the ^{2}H label of brassinin (99%) into spirobrassinin (81%) and cyclobrassinin (75%) was realized, as described above. These results could be explained if we assume neighboring group participation by the hydroxyl group in **4** and also the presence of methanethiol or its equivalent in the water phase.

Origin of the Indole Ring of Brassinin. It is known that glucosinolates are biosynthesized through a common biosynthetic pathway from an amino acid precursor.[19] In the case of indole glucosinolate, glucobrassicin (**9a**), it is biosynthesized from L-tryptophan.[20] Therefore, brassinin would be derived from L-tryptophan, though it is not clear whether the indole or indole related phytoalexins are biosynthesized from indole glucosinolates or directly from tryptophan. In order to confirm the origin of an indole moiety of **1a**, incorporation of L-tryptophan was examined.

When L-[4′-^{2}H]tryptophan (4′-$^2H_3$, 80%)[21] was fed to the UV-elicited turnip tissue and incubated for 25 h, **1a** and **3** were isolated as main metabolites. The ^{1}H-NMR spectrum of **3** showed a small decrease (18%) in the aromatic methine signal at C4, indicating the incorporation of the labeled L-tryptophan. To confirm the presence of ^{2}H at C4 in spirobrassinin, the ^{2}H-NMR was measured. Figure 3 shows the ^{2}H-NMR spectrum of [4-^{2}H]-spirobrassinin. A clear signal in the aromatic region indicated unambiguously the incorporation of ^{2}H into the benzene ring of **3**. However, a clear ^{2}H-NMR spectrum was not obtained in the case of **1a**, because of a scanty amount of available **1a**. Since the previous feeding experiments established that **3** is biosynthesized from **1a**, the indole ring of **1a** must originate from L-tryptophan.

Involvement of a Molecular Rearrangement in the Pathway from L-Tryptophan to Brassinin. The crucial point in the biosynthetic pathway leading to **1a** is whether the thiocarbonyl carbon of **1a**, therefore, the relevant imino carbon in **3**, originates from the C2 carbon of L-tryptophan (Scheme 2). If this is the case, the biosynthetic pathway to **1a** should involve a molecular rearrangement step since the thiocarbonyl carbon of **1a** is separated from the methylene carbon by a nitrogen atom. To test this possibility, a feeding experiment with DL-[2-^{13}C]-tryptophan was carried out. Administration of the labeled tryptophan resulted in a 4-fold enhancement of the imino carbon NMR signal of **3** at δ 164 (Figure 4). This result indicates the involvement of a molecular rearrangement in the pathway from tryptophan to **1a** and is suggestive of indol-3-ylmethyl isothiocyanate (**10a**) as a key intermediate to **1a**. Although less labile (1-methoxyindol-3-yl)methyl isothiocyanate (**10b**) has been recently detected by mass spectrometry in the enzymatic hydrolysis products of neoglucobrassicin (**9b**),[22] the unstable isothiocyanate (**10a**) has not been isolated to date. Only degradation products such as 3-indoleacetonitrile and 3-indolemethanol have been reported (Scheme 1).[23]

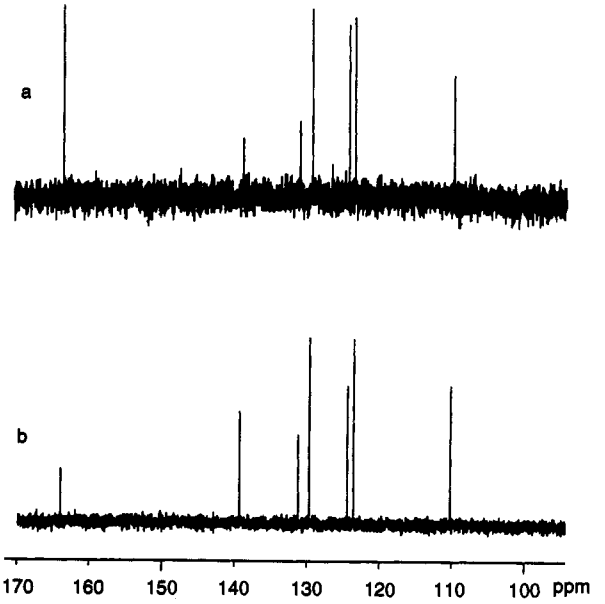
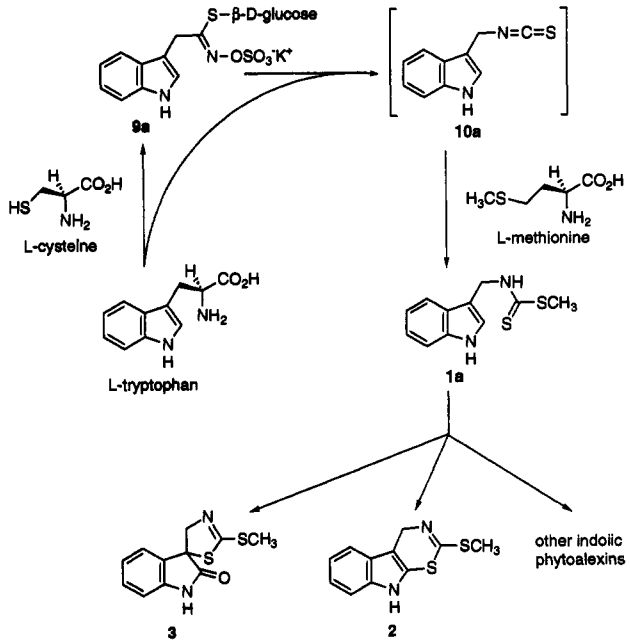


Figure 4. Partial proton noise decoupled 67.5-MHz ^{13}C-NMR spectra of spirobrassinin (**3**): (a) after incorporation of [2-^{13}C]tryptophan; (b) natural abundance.

Scheme 2. Biosynthetic Pathway of Cruciferous Phytoalexins



The Isothiocyanate Intermediate. In order to detect the labile indol-3-ylmethyl isothiocyanate (**10a**), a trapping experiment with

(19) (a) Dewick, P. M. *Natl. Prod. Rep.* **1984**, *1*, 545–549. (b) Glendening, T. M.; Poulton, J. E. *Plant Physiol.* **1988**, *86*, 319–321.

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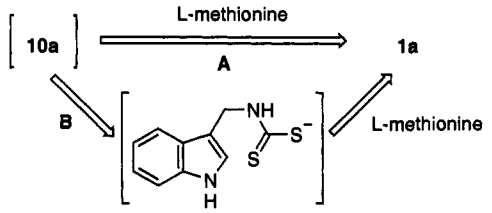
Scheme 3. Direct and Stepwise Mechanisms of Methylthiolation to Brassinin (**1a**)



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methanethiolate was attempted. Turnip roots were homogenized in a blender with 15% aqueous sodium methanethiolate in phosphate buffer (pH 7.0) for 5 min, and the homogenate was extracted immediately with ethyl acetate. Separation of the extract gave two adducts. Spectral data and direct comparison with the authentic samples revealed that they were **1a** and $PhCH_2$-CH_2-NH-CS-SCH_3, which was named phenethylbrassinin (**13**).

Neither **1a** nor phenethylbrassinin (**13**) was isolated in the absence of sodium methanethiolate. Isolation of **13** is in accord with the previous analytical studies of glucosinolates,[5a] where large amounts of a corresponding glucosinolate, gluconasturtiin, were detected in the turnip tissue. The present isolation of **1a** suggests transient formation of **10a** as a reaction intermediate.

To examine a possible role of isothiocyanates in the biosynthesis of cruciferous phytoalexins, moderately reactive benzyl isothiocyanate was chosen as a model substrate and administered to the turnip tissue without sodium methanethiolate. A new metabolite named benzylbrassinin (**14**) was isolated and identified as $PhCH_2$-NH-CS-SCH_3 by direct comparison with a synthetic sample.

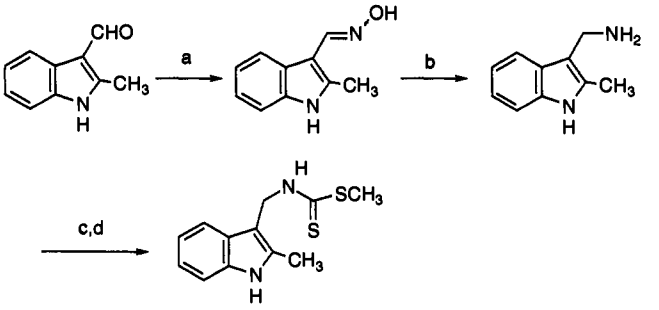
Formation of **14** in the turnip tissue indicates that isothiocyanate **10a** is involved in the biosynthesis of **1a**. It remains to be proved whether the isothiocyanate **10a** can be formed *via* hydrolysis of **9a** and/or directly from L-tryptophan, e.g. *via* thiohydroxamic acid.

Intact Incorporation of the Methylthio Group of Methionine. To confirm the origin of the methyl group in brassinin (**1a**), an incorporation study of L-[*methyl*-$^2H_3$]methionine was carried out. The ^{1}H-NMR spectra of isolated **1a**, **2**, and **3** indicated unambiguously that each methyl group of **1a**, **2**, and **3** arose from L-methionine. Remarkable incorporation of the methyl group was evident from a decrease of respective methyl signal in each ^{1}H-NMR spectrum: **1a** 35%, **2** 34%, and **3** 21%. These results indicated that methylation occurred in a late stage of the biosynthesis. This agrees with our proposal that **1a** is biosynthesized *via* isothiocyanate **10a**.

The methyl group of brassinin is derived from the methyl group of L-methionine, as described above. There are two possible biosynthetic routes from unstable isothiocyanate **10a** to **1a** (Scheme 3). Path A involves a direct attack by a methylthio group from L-methionine, whereas path B is a stepwise mechanism *via* a dithiocarbamic acid intermediate (**15**) or its equivalent.

In order to clarify whether the methylthio group in the phytoalexin is introduced directly or in a stepwise manner, a mixture of L-[*methyl*-$^3H_3$]methionine and L-[^{35}S]methionine was fed to the UV-elicited turnip tissue. The mixture is considered doubly labeled L-[*methyl*-$^3H_3$, ^{35}S]methionine, provided a negligible isotope effect exists. The radioactive ratio of $^{35}S/^3H$ in the L-methionine mixture was 2.4. Forty eight hours after administration of the mixture, metabolites were extracted with ethyl acetate from the aqueous phase and separated to yield 0.2 mg of **1a** and 2.4 mg of **3**. The ratio of $^{35}S/^3H$ of isolated **1a** and **3** was 2.5 and 2.2 at several concentrations, respectively. Therefore, the ratio is considered unchanged during biosynthesis of **1a** and **3**. This result indicates unambiguously that **1a** is biosynthesized by intact incorporation of the methylthio group from L-methionine to the isothiocyanate **10a**. The same results were obtained when several experiments were done using L-methionine with different ratios of $^{35}S/^3H$.

Scheme 4. Preparation of 2-Methylbrassinin (**16**)[a]



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[a] Reagents and conditions: (a) 1.6 equiv of NH_2OH·HCl, Na_2CO_3, H_2O-C_2H_5OH, reflux, 16 h, 75%; (b) Devarda's alloy, 1 M NaOH, H_2O-CH_3OH, 25 °C, 20 min; (c) 2.7 equiv of CS_2, triethylamine, dry pyridine, 0 °C, 1 h; (d) 2.5 equiv of CH_3I, 25 °C, 18 h, 72% from oxime.

This intact incorporation of the methylthio group of L-methionine is unusual since many natural products with the methylthio group are derived by methylation of thiols,[24] which originate from the cysteine or glutathione conjugate of each acceptor molecule.

Since the two sulfur atoms of glucobrassicin (**5**) are derived from L-cysteine,[25] the sulfur atom of the isothiocyanate intermediate (**10a**) and the thiocarbonyl sulfur atom of **1a** are most likely derived from L-cysteine.

To confirm the origin of the other sulfur atom, feeding experiments with L-[^{35}S]cysteine were examined. The radioactivities of isolated **1a** and **3** were clearly high. These results support the conclusion that the thiocarbonyl sulfur atom originates from L-cysteine.

An Intermediate Connecting Brassinin with Other Indole Phytoalexins. In a previous section, it was shown that **2** and **3** are biosynthesized from an advanced precursor **1a** and that labeled **2** and **4** are not incorporated into **3**. These results suggest that **2** and **3** are derived independently from **1a** by way of a common intermediate. Production of a variety of indole or indole related phytoalexins can be explained by postulating a common intermediate which links **1a** and other cruciferous phytoalexins.

To understand the intermediate, 2-methylbrassinin (**16**) was chosen as a probe. Since the compound **16** is blocked by a methyl group at the C2 position, the formation of a cyclobrassinin-type or a spirobrassinin-type compound is structurally prohibited. Therefore, possible isolation of a corresponding intermediate was expected. 2-Methylbrassinin was synthesized from 3-formyl-2-methylindole by the same procedure used for **1a** (Scheme 4). The model substrate **16** was administered to the UV-elicited turnip tissue. After a 27-h incubation, two new compounds, **17** (4 mg) and **18** (3 mg), were isolated (Scheme 5). Both compounds were not detected in the control tissue.

Compound **17**, $[\alpha]^{25}_D = -21.8°$, has a molecular formula of $C_{12}H_{12}N_2S_2$. The UV spectrum (λ_{max} (CH_3OH) 227 and 299(sh) nm)) suggests that it has an indolenine structure.[26] The ^{1}H-NMR spectrum shows the presence of four successive aromatic protons (δ 7.26 (1H, ddd, J = 7.3, 7.3, and 1.5 Hz), 7.37 (1H, ddd, J = 7.3, 7.3, and 1.1 Hz), 7.45 (1H, br d, J = 7.3 Hz), 7.53 (1H, br d, J = 7.3 Hz)), one methylthio group (δ 2.32 (3H, s)), one benzylic methyl group (δ 2.66 (3H, s)), and two methylene protons (δ 4.36 (1H, d, J = 15.8 Hz), 4.47 (1H, d, J = 15.8 Hz)). The coupling pattern (d, J = 15.8 Hz) of the methylene protons is suggestive of a methylene group attached to a spiro ring, as in

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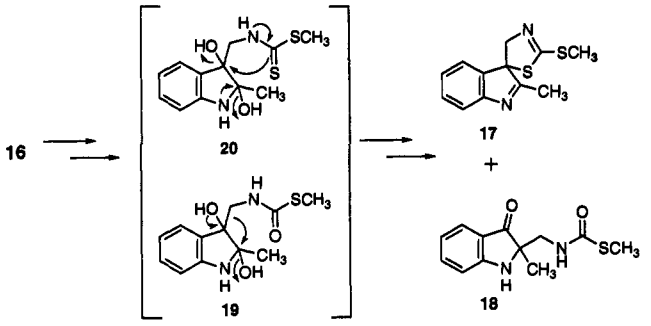
Scheme 5. Mechanisms of Production of Compounds **17** and **18**



3. The IR absorption (ν_{max} 1577 cm^{-1}) indicates the presence of a >C=N- group. These spectral data and eight degrees of unsaturation indicate that the product **17** is a tricyclic compound with a spiro ring system. An EI-MS fragment at *m/z* 175 (base peak, $(M - -N{=}C{-}SCH_3)^+$) supports the partial structure $-N{=}C{-}SCH_3$. The full structure is therefore represented by formula **17**. Results of difference NOE experiments are consistent with the proposed structure **17**.

Compound **18**, $[\alpha]^{25}_D = -9.2°$, has a molecular formula of $C_{12}H_{12}N_2OS$. Its ^1H- and ^{13}C-NMR spectra also show the presence of four successive aromatic protons (δ_H 6.84 (1H, dd, J = 7.8 and 7.3 Hz), 6.88 (1H, d, J = 8.3 Hz), 7.47 (1H, ddd, J = 8.3, 7.3, and 1.5 Hz), 7.58 (1H, d, J = 7.8 Hz), δ_C 112.9 (d), 119.3 (d), 124.8 (d), 137.7 (d)), one methyl group (δ_H 1.33 (3H, s), δ_C 20.8 (q)), one methylthio group (δ_H 2.31 (3H, s), δ_C 12.5 (q)), two methylene protons (δ_H 3.56 (2H, d, J = 5.9 Hz) δ_C 47.1 (t)), two D_2O exchangeable protons (δ_H 5.02 (1H, br s), 5.68 (1H, br s, t-like, not completely exchangeable with D_2O)), and one sp^3 quaternary carbon atom (δ_C 29.7 (s)). The UV spectrum (λ_{max} (CH_3OH) 254(sh) and 391 nm) indicates that it must have an indoxyl nucleus.[27] These results suggest that the side chain of 2-methylbrassinin (**16**) rearranges to the C2 carbon atom of the indoxyl nucleus. The high-field shift of the methyl signal in the ^1H-NMR spectrum (from δ 2.64 in **16** to δ 1.33 in **18**) and the presence of one sp^3 quaternary carbon atom also support this indoxyl structure. Results of difference NOE experiments suggest that the methylene and one of the methyl groups are connected to the same C2 carbon atom. ^1H–^1H decoupling experiments indicate that the methylene is connected to a >NH group. The EI-MS spectrum of **18** does not show a molecular ion peak. However the FD-MS spectrum clearly indicates the presence of a molecular ion at *m/z* 250. Therefore, the residual side chain at C2 must include a thiocarbamate moiety. Hence, the structure of the second product is represented by formula **18**.

These two metabolites, especially the compound **18**, give important information pertaining to the intermediate in question. Formation of indoxyl (**18**) suggests that the immediate precursor to **18** is diol intermediate **19**, which gives rise to **18** in a pinacol-type rearrangement (Scheme 5). The thiocarbamate side chain of **19** may be derived from the corresponding dithiocarbamate by oxidation. The supposed structure of diol intermediate **20** could account for the formation of metabolite **17**. Dehydration of **20**, followed by internal displacement could lead to spiro compound **17**. Furthermore, the carbonyl group of thiocarbamate **19** is less nucleophilic as compared with the thiocarbonyl group of **20**. Therefore, rearrangement would precede cyclization in diol **19**.

Although epoxide **21** has the same oxidation state as diol **20**, the latter is preferred as a more direct precursor because of the presence of an oxygen atom at the C2 position in spirobrassinin and in dioxibrassinin. Furthermore, the structures of **22** and **23**, isolated from Japanese radish as minor phytoalexins,[28] support

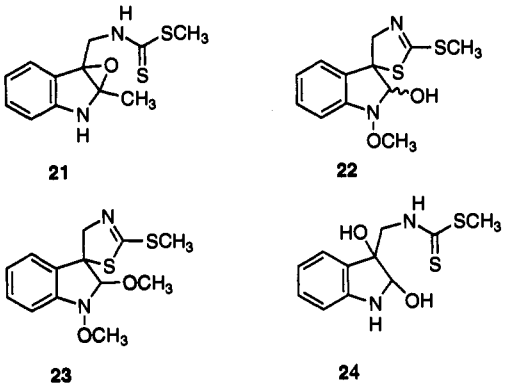


Figure 5.

the presence of a corresponding diol intermediate. That is, compound **22** could be biosynthesized by cyclization of the corresponding diol, which would be derived from methoxybrassinin (**1b**). Further methylation or methanolysis of **22** could yield compound **23**. Isolation of the alcohol **22** could be explained by its resistance to further oxidation. Steric hindrance induced by the *N*-methoxyl group would be one reason for this resistance.

When the concept of a diol intermediate is applied to **1a**, the corresponding diol intermediate **24** can be proposed (Figure 5). This diol precursor, **24**, can explain the formation of a variety of indole or indole related phytoalexins. For example, internal displacement at the C2 carbon followed by dehydration can lead to cyclobrassinin (**2**), and oxidation of the hydroxyl group at C2 of diol **24** can give **4**. Internal displacement of the hydroxyl at C3 followed by oxidation of the secondary hydroxyl group would yield **3**. This biosynthetic pathway is consistent with the negative incorporation of **2** and **4** to **3**.

Discussion

The biosynthetic pathway of cruciferous phytoalexins is summarized in Scheme 2. The fundamental phytoalexin, brassinin (**1a**), is biosynthesized and metabolized as follows. It originates from L-tryptophan with incorporation of one sulfur atom from L-cysteine, leading to the unstable intermediate, isothiocyanate **10a**, which is directly methylthiolated to yield **1a**. Brassinin (**1a**) is metabolized to **2**, **3**, and other indolic phytoalexins *via* an unstable oxidized intermediate.

The intact incorporation of the methylthio group from L-methionine to isothiocyanate **10a** suggests that brassinin could be produced by nonenzymatic addition of methanethiol produced from L-methionine to the isothiocyanate. Although formation of methanethiol from broccoli under anaerobic conditions has been reported by Forney et al.,[29] detection by gas chromatography of methanethiol from UV-irradiated turnip tissue has not been successful thus far. Since methylthio groups in natural products are generally derived stepwise from methyl donors and sulfur donors, the direct methylthiolation is noteworthy. The direct methylthiolation also supports the presence of the labile intermediate, indole-3-ylmethyl isothiocyanate.

Methoxybrassinin (**1b**) is a representative of N-methoxylated cruciferous phytoalexins. This phytoalexin belongs to a rare group of *N*-methoxyindoles found in nature. It is not clear at the present stage whether **1b** is derived from neoglucobrassicin (**9b**) or from **1a** *via* N-hydroxylation followed by methylation. Although an incorporation experiment of labeled **1a** to **1b** has been undertaken, no incorporation has been observed so far.

Another type of cruciferous phytoalexins, brassilexin (**5**), has recently been isolated from Indian mustard (*Brassica nigra*),[7a] but has not been isolated from our materials to date. Since brassilexin lacks a methylthio group, correlation between brassilexin and brassinin is not obvious. However, Barbier et al. have

(27) Sundberg, R. J.; Yamazaki, T. *J. Org. Chem.* **1967**, *32*, 290–294.
(28) ^1H-NMR, IR, low- and high-resolution EI-MS, and UV are included as supplementary material. Monde, K.; Takasugi, M.; Shirata, A. Detailed results are in preparation for manuscript.
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demonstrated that brassilexin is derived chemically from cyclobrassinin by $NaIO_4$ oxidation.[30] According to their report, **5** is produced from cyclobrassinin disulfoxide,[7b] which in turn is derived from **2**. The co-occurrence of cyclobrassinin sulfoxide and **2** in the same crucifer[7b] suggests that **5** is derived from **2** *via* cyclobrassinin sulfoxide and therefore from **1a**, although no experimental evidence is available.

In the present paper, diol **24** is postulated as a key intermediate in the biosynthetic pathway to account for the formation of **2**, **3**, and other related phytoalexins. Although such a diol intermediate has been proposed by Scott in the biosynthetic studies of indole alkaloids,[31] no experimental evidence for the diol intermediate has been presented. For example, it is possible that diol intermediate **24** is formed by a zwitterion followed by oxygen uptake and reduction of the ion, by enzyme-catalyzed dioxetane formation followed by reductive opening of the dioxetane ring,[32] or by β-hydroxyindolenine formation followed by hydration.

The proposed biosynthetic pathway of the cruciferous phytoalexins indicates that a close relationship exists between the indole phytoalexins and indole glucosinolates. Time course studies on the sliced turnip tissue[5a] show that the levels of both indole glucosinolates and phytoalexins increase significantly after UV irradiation, whereas only the former increases in nonirradiated control tissue. These results imply that the accumulated indole glucosinolates are converted to their respective isothiocyanate and then to **1a** or **2** by methylthiolation through a UV-induced metabolic pathway. It is interesting to note that an increase in the levels of indole glucosinolates is accompanied by a decrease in the levels of aliphatic glucosinolates. Massive accumulation of the indole glucosinolates as well as a decrease in aliphatic glucosinolates has also been observed in wounded or insect-infected plant tissue.[33] These observations lead to the conclusion that wounded crucifers provide against possible microbial invasion an accumulation of indole glucosinolates, which serves as precursors of indole phytoalexins at the sacrifice of aliphatic glucosinolates. When the crucifers are exposed to pathogens, they produce indolic phytoalexins rapidly by using the accumulated indole glucosinolates.

Experimental Section

General Procedures. The ^{1}H-NMR (90 and 400 MHz) and ^{13}C-NMR (100.6 MHz) spectra were obtained on JEOL JNM-EX90, JNM-EX400, and JNX-GX400 spectrometers. Chemical shifts are given in parts per million (ppm) relative to the TMS scale by reference to the solvent signal. IR spectra were recorded on a JASCO IR-700 spectrometer. UV spectra were obtained using a JASCO Ubest-30 spectrophotometer. Optical rotation measurements were done on a JASCO DIP-140 polarimeter. Low- and high-resolution EI-MS spectra were obtained using a JEOL JMS-DX300 spectrometer. FD-MS spectra were recorded using a JEOL JMS-01SG-2 spectrometer. Melting points were determined by a Yanagimoto MP micro melting point apparatus and were uncorrected. The HPLC analyses were performed on a JASCO HPLC system equipped with a Model 801-SC system controller, a Model 851-AS automatic sampler, a Model 880-50 line degasser, a Model 880-02 low-pressure gradient unit, a Model 865-CO column oven, a Model 880-PU pump, and a Model UVIDEC-100-V UV detector. A Shimadzu Chromatopac C-R6A integrator was used for measuring peak areas. Analytical conditions were used as previously described.[18] Reagents and solvents were obtained from Nacalai tesque, Aldrich Chemical Co., and Wako Pure Chemical Industries. All chemicals of reagent grade were used without further purification unless otherwise noted. L-[4′-^{2}H]Tryptophan (80%) was synthesized from L-tryptophan and D_2O (99%) by a reaction reported by Saito et al.[21] [*methyl*-$^2H_3$]Methyl iodide (99%), [*methyl*-$^2H_3$]methionine (99%), and DL-[2-^{13}C]tryptophan (99%) were purchased from E. Merck AG., Aldrich Chemical Co., and MSD Isotope, respectively. L-[*methyl*-$^3H_3$]Methionine, L-[^{35}S]methionine, and L-[^{35}S] cysteine were purchased from NEN Research Products.

Feeding Experiments with Labeled Precursors. Turnip roots (*B. campestris* L. ssp. *rapa* cv. Shirokamome) were cut horizontally, and hemispherical holes (2 cm in diameter) were made on each surface. After 12–24 h of incubation at 25 °C in moist plastic cases covered loosely with special polyethylene film (Aisaika film), the tissues were irradiated with a 15-W germicidal lamp for 10 min and incubated for an additional 6–12 h. Each hole was then filled with 0.1% Tween-80 aqueous solution (or milky suspension) containing a sample at a concentration of 1–3 mmol dm^{-3}. When the labeled compound could not be dissolved in Tween-80 aqueous solution, the compound was dissolved in small amounts of dimethyl sulfoxide (DMSO), and the DMSO solution was added carefully to the 0.1% Tween-80 aqueous solution with stirring (Figure 2).

Labeled compound was incorporated into the tissue to yield metabolites which diffused into the aqueous phase. The turnip tissue was incubated for the period indicated by HPLC analysis of sample aliquots. The aqueous phase was harvested at the optimum time and extracted with ethyl acetate. The extracts were separated by silica gel column chromatography and by preparative TLC on silica gel to give labeled metabolite(s).

Incorporation of [*methyl*-$^2H_3$]Brassinin. Sample solutions were prepared as follows. Synthetic [*methyl*-$^2H_3$]brassinin (99%) was dissolved in 1.6 mL of dimethyl sulfoxide (DMSO), and then the DMSO solution was carefully diluted with 850 mL of 0.1% Tween-80 aqueous solution with vigorous shaking. The resulting milky solution was used in the experiment. Each hole was filled with the 0.1% Tween-80 aqueous solution containing [*methyl*-$^2H_3$]brassinin at a concentration of 1.0 mmol dm^{-3}. Totally, 850 mL of sample solution was added. The tissues were then incubated for 37 h. The combined aqueous phase was extracted twice with 200 mL of ethyl acetate. The combined extract was dried over Na_2SO_4 and evaporated *in vacuo*. The residue (84 mg) was separated by silica gel column chromatography (methanol–dichloromethane, 1:99), followed by preparative TLC on silica gel to give **2** (2 mg) and **3** (11 mg).

2: ^{1}H-NMR (400 MHz, $CDCl_3$) δ 2.56 (0.75H, s), 5.09 (2H, d, J = 4 Hz), 7.14 (1H, ddd, J = 7, 7, and 2 Hz), 7.18 (1H, ddd, J = 7, 7, and 2 Hz), 7.33 (1H, dd, J = 7 and 2 Hz), 7.49 (1H, dd, J = 7 and 2 Hz), and 7.91 (1H, br s).

3: ^{1}H-NMR ($CDCl_3$) δ 2.62, (0.57H, s), 4.51 (1H, d, J = 15.1 Hz), 4.68 (1H, d, J = 15.1 Hz), 6.92 (1H, d, J = 7.8 Hz), 7.09, (1H, ddd, J = 6.8, 7.8, and 1.0 Hz), 7.26 (1H, ddd, J = 7.8, 7.8, and 1.5 Hz), 7.36 (1H, d, J = 6.8 Hz), and 8.61 (1H, br s, D_2O exchangeable).

Incorporation of [*methyl*-$^2H_3$]Cyclobrassinin. Synthetic [*methyl*-$^2H_3$]-cyclobrassinin (99%, 60 mg) was dissolved in 0.5 mL of DMSO, and the DMSO solution was carefully diluted with 0.1% Tween-80 aqueous solution with vigorous shaking. The resulting milky solution was used in the experiment. Each hole was then filled with the 0.1% Tween-80 aqueous solution containing [*methyl*-$^2H_3$]cyclobrassinin at a concentration of 0.84 mmol dm^{-3}. Totally, 300 mL of sample solution was added. The tissues were then incubated for 32 h. The combined aqueous phase was extracted twice with 200 mL of ethyl acetate, and the combined extract was dried over Na_2SO_4 and evaporated *in vacuo*. The residue (53 mg) was separated by silica gel column chromatography (methanol–dichloromethane, 1:99), followed by preparative TLC on silica gel to give **3** (5 mg).

3: ^{1}H-NMR ($CDCl_3$) δ 2.62, (3H, s), 4.51 (1H, d, J = 15.1 Hz), 4.68 (1H, d, J = 15.1 Hz), 6.92 (1H, d, J = 7.8 Hz), 7.09, (1H, ddd, J = 6.8, 7.8, and 1.0 Hz), 7.26 (1H, ddd, J = 7.8, 7.8, and 1.5 Hz), 7.36 (1H, d, J = 6.8 Hz), and 8.61 (1H, br s, D_2O exchangeable).

Incorporation of DL-[*methyl*-$^2H_3$]Dioxibrassinin. Synthetic DL-[*methyl*-$^2H_3$]dioxibrassinin (99%, 105 mg) was dissolved with 1.0 mL of DMSO. Then the DMSO solution was carefully diluted with 500 mL of 0.1% Tween-80 aqueous solution with vigorous shaking. The resulting milky solution was poured into each hole at a concentration of 0.77 mmol dm^{-3}. Totally, 500 mL of sample solution was added. The tissues were then incubated for 30 h, and the combined aqueous phase was extracted twice with 500 mL of ethyl acetate. The combined extract was dried over Na_2SO_4 and evaporated *in vacuo*. The residue (150 mg) was separated by silica gel column chromatography (methanol–dichloromethane 1:99), followed by preparative TLC on silica gel to give **3** (10 mg) and recovered **4** (43 mg). Both compounds were identified by ^{1}H-NMR, EI-MS, and HPLC retention time. The ^{1}H-NMR of isolated **3** from the aqueous solution revealed no incorporation of synthetic ^{2}H-labeled **4** to **3**.

3: ^{1}H-NMR ($CDCl_3$) δ 2.62, (3H, s), 4.51 (1H, d, J = 15.1 Hz), 4.68 (1H, d, J = 15.1 Hz), 6.92 (1H, d, J = 7.8 Hz), 7.09, (1H, ddd, J = 6.8,

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(33) Birch, A. N. E.; Giffiths, D. W.; Smith, W. H. M. *J. Sci. Food Agric.* **1990**, *51*, 309–320.

7.8, and 1.0 Hz), 7.26 (1H, ddd, J = 7.8, 7.8, and 1.5 Hz), 7.36 (1H, d, J = 6.8 Hz), and 8.61 (1H, br s, D_2O exchangeable).

4 (recovered after 6-h incubation): ^{1}H-NMR (acetone-d_6) δ 2.70 (0.87H, s), 4.00 (1H, dd, J = 14.2 and 3.9 Hz), 4.53 (1H, dd, J = 14.2 and 5.9 Hz), 5.58 (1H, br s), 7.06 (1H, d, J = 7.6 Hz), 7.15 (1H, dd, J = 7.6 and 7.6 Hz), 7.40 (1H, dd, J = 7.6 and 7.6 Hz), 7.55 (1H, d, J = 7.6 Hz), 8.97 (1H, br s), and 9.63 (1H, br s).

The tissues around the holes were also cut off and homogenized in a Waring blender with 1 L of ethyl acetate. The homogenized tissue was separated from the organic phase by centrifugation, and the resulting ethyl acetate layer was dried over Na_2SO_4 and evaporated *in vacuo*. The residue (653 mg) was separated by successive rounds of column chromatography, followed by preparative TLC on SiO_2 to give **3** (9 mg). The ^{1}H-NMR spectrum of **3** isolated from turnip tissue also revealed the same result as that from the aqueous solution.

Incorporation of L-[4′-^{2}H]Tryptophan. Each hole was filled with 0.1% Tween-80 aqueous solution (450 mL) containing L-[4′-^{2}H]tryptophan (80%) at a concentration of 1.2 mmol dm^{-3}. After 14 h, the aqueous solution was harvested, and then additional aqueous solution (400 mL) of L-[4′-^{2}H]tryptophan (0.8 mmol dm^{-3}) was added. Totally, 176 mg of L-[4′-^{2}H]tryptophan was added. The tissues were then incubated for 11 h. The combined aqueous solution was extracted twice with 600 mL of ethyl acetate, and the extract was dried over Na_2SO_4 and evaporated *in vacuo*. The residue (129 mg) was separated by silica gel column chromatography (methanol–dichloromethane, 1:99), followed by preparative TLC on silica gel to give **1a** (2 mg) and **3** (11 mg).

1a: ^{1}H-NMR ($CDCl_3$) δ 2.65 (3H, s), 5.07 (2H, d, J = 4 Hz), 7.0 (1H, br s), 7.18 (1H, ddd, J = 8, 8, and 1 Hz), 7.25 (1H, ddd, J = 8, 8, and 1 Hz overlapped singlet 1H), 7.42 (1H, d, J = 8 Hz), 7.64 (0.95H, br d, J = 8 Hz), and 8.16 (1H, br s).

3: ^{1}H-NMR ($CDCl_3$) δ 2.62, (2.46H, s), 4.51 (1H, d, J = 15.1 Hz), 4.68 (1H, d, J = 15.1 Hz), 6.92 (1H, d, J = 7.8 Hz), 7.09, (1H, ddd, J = 6.8, 7.8, and 1.0 Hz), 7.26 (1H, ddd, J = 7.8, 7.8, and 1.5 Hz), 7.36 (0.82H, d, J = 6.8 Hz), and 8.61 (1H, br s, D_2O exchangeable); EI-MS m/z (%) 251 (M^+ + 1, 11.1), 250 (M^+, 36.1), 204 ($M^+ - SCH_3 + 1$, 14.7), 203 ($M^+ - SCH_3$, 56.6), 179 ($M^+ - C_2H_3NS + 2$, 7.8), 178 ($M^+ - C_2H_3NS + 1$, 25.7), and 177 ($M^+ - C_2H_3NS$, 100).

Incorporation of DL-[2-^{13}C]Tryptophan. Each hole was filled with 0.1% Tween-80 aqueous solution (400 mL) containing DL-[2-^{13}C]tryptophan (99%, 98 mg) at a concentration of 1.2 mmol dm^{-3}. The tissues were then incubated for 38 h. The aqueous solution was extracted twice with 400 mL of ethyl acetate. The combined extract was dried over Na_2SO_4 and evaporated *in vacuo*. The residue (67 mg) was separated by silica gel column chromatography (methanol–dichloromethane, 1:99), followed by preparative TLC on silica gel to give **1a** (2 mg), **2** (0.4 mg), and **3** (3 mg). Each compound was identified by comparison of its ^{1}H-NMR spectrum and HPLC retention time with those of authentic samples.

3: ^{13}C NMR (100.6 MHz, $CDCl_3$, COM) δ 15.7, 64.6, 75.1, 110.1, 123.7, 124.6, 130.0, 131.3, 139.3, and 164.1.

Incorporation of L-[*methyl*-^{2}H$_3$]Methionine. Each hole was filled with 0.1% Tween-80 aqueous solution (500 mL) containing L-[*methyl*-^{2}H$_3$]-methionine (99%) at a concentration of 2.0 mmol dm^{-3} and nonlabeled L-tryptophan at a concentration of 3.9 mmol dm^{-3}. After 24 h, the aqueous solution was harvested, and additional aqueous solution (500 mL) of L-[*methyl*-^{2}H$_3$]methionine (4.0 mmol dm^{-3}) and non-labeled L-tryptophan (3.9 mmol dm^{-3}) was added. Totally, 456 mg of L-[*methyl*-^{2}H$_3$]-methionine was added. The tissues were further incubated for 32 h. The combined aqueous solution was extracted twice with 500 mL of ethyl acetate. The combined extract was dried over Na_2SO_4 and evaporated *in vacuo*. The residue (189 mg) was separated by silica gel column chromatography (methanol–dichloromethane, 1:99), followed by preparative TLC on silica gel to give **1a** (2 mg), **2** (1 mg), and **3** (7 mg).

1a: ^{1}H-NMR ($CDCl_3$) δ 2.65 (1.95H, s), 5.07 (2H, d, J = 4 Hz), 7.0 (1H, br s), 7.18 (1H, ddd, J = 8, 8, and 1 Hz), 7.25 (1H, ddd, J = 8, 8, and 1 Hz overlapped singlet 1H), 7.42 (1H, d, J = 8 Hz), 7.64 (1H, br d, J = 8 Hz), and 8.16 (1H, br s).

2: ^{1}H-NMR ($CDCl_3$) δ 2.56 (1.98H, s), 5.09 (2H, d, J = 4 Hz), 7.14 (1H, ddd, J = 7, 7, and 2 Hz), 7.18 (1H, ddd, J = 7, 7, and 2 Hz), 7.33 (1H, dd, J = 7 and 2 Hz), 7.49 (1H, dd, J = 7 and 2 Hz), and 7.91 (1H, br s).

3: ^{1}H-NMR ($CDCl_3$) δ 2.62, (2.37H, s), 4.51 (1H, d, J = 15.1 Hz), 4.68 (1H, d, J = 15.1 Hz), 6.92 (1H, d, J = 7.8 Hz), 7.09, (1H, ddd, J = 6.8, 7.8, and 1.0 Hz), 7.26 (1H, ddd, J = 7.8, 7.8, and 1.5 Hz), 7.36 (1H, d, J = 6.8 Hz), and 8.61 (1H, br s, D_2O exchangeable).

Incorporation of Benzyl Isothiocyanate. Each hole was filled with 0.1% Tween-80 aqueous solution containing benzyl isothiocyanate (Nacalai tesque) at a concentration of 10 mmol dm^{-3}. Totally, 450 mL of sample solution was added. The tissues were then incubated for 24 h. The aqueous phase was extracted twice with 500 mL of ethyl acetate. The combined extract was dried over Na_2SO_4 and evaporated *in vacuo*. The residue (268 mg) was separated by silica gel column chromatography (benzene–hexane, 1:1) to give 3 mg of benzylbrassinin (**14**), which was identified by direct comparison with synthetic **14**.

Incorporation of a Mixture of L-[*methyl*-^{2}H$_3$]- and L-[^{35}S]Methionine. Each hole was filled with 1.0 mM cold L-methionine and 0.1% Tween-80 aqueous solution containing a mixture of L-[*methyl*-^{3}H$_3$]methionine and L-[^{35}S]methionine (^{3}H, 11.1 kBq/μmol; ^{35}S, 82.9 kBq/μmol). Totally, 300 mL of sample solution was added. The tissues were then incubated for 48 h at 25 °C. The combined aqueous phase was extracted twice with 300 mL of ethyl acetate, and the combined extract was dried over Na_2SO_4 and evaporated *in vacuo*. The residue (30 mg) was separated twice by preparative TLC on silica gel to give labeled **1a** (0.2 mg; R_f 0.53–0.55; methanol–dichloromethane, 2:98) and **3** (2 mg; R_f 0.13–0.16, methanol–dichloromethane, 2:98; R_f 0.47–0.54; benzene–ethyl acetate, 1:1), after analyzing TLC with AMBIS 1000 radioanalytic imaging system (AMBIS). Radioactivity measurements were carried out on an Aloka LSC-3500 liquid scintillation counter with scintillation cocktail (Scintisol EX-H, Dojindo Laboratories).

Incorporation of L-[^{35}S]Cysteine. Each hole was filled with 1.0 mM cold L-cysteine in 0.1% Tween-80 aqueous solution containing L-[^{35}S]-cysteine (127 kBq/μmol). Totally, 700 mL of the sample solution was added. The tissues were then incubated for 48 h at 25 °C. The combined aqueous phase was extracted twice with 300 mL of ethyl acetate. The combined extract was dried over Na_2SO_4 and evaporated *in vacuo*. The residue (52 mg) was separated twice by preparative TLC on silica gel to give labeled **1a** (1 mg; R_f 0.53–0.55; methanol–dichloromethane, 2:98) and **3** (3 mg; R_f 0.13–0.16; methanol–dichloromethane, 2:98; R_f 0.47–0.54; benzene–ethyl acetate, 1:1), after analyzing TLC with AMBIS 1000 radioanalytic imaging system. Radioactivity measurements were carried out on an Aloka LSC-3500 liquid scintillation counter with scintillation cocktail.

Incorporation of 2-Methylbrassinin (16). Each hole was filled with 0.1% Tween-80 aqueous solution of synthetic 2-methylbrassinin (**16**) with 400 μL of DMSO (0.48 mmol dm^{-3}, 700 mL). The tissues were then incubated for 27 h at 20 °C. The ethyl acetate extracts (2 × 500 mL) of the above aqueous solution were dried over Na_2SO_4. The solvent was evaporated *in vacuo*, and the residue (140 mg) was separated by silica gel column chromatography (methanol–dichloromethane, 1:99), followed by preparative TLC on silica gel to give 2-methyl-3-formylindole (2 mg), compound **17** (4 mg), and **18** (3 mg).

Compound 17: $C_{12}H_{12}N_2S_2$ (m/z 175.0466, M^+), gum; $[\alpha]^{18}_D$ = −21.76° (c 0.38, $CHCl_3$); IR ($CHCl_3$) 2926, 2852, 1577 (st), 1457, 1429, 1377, 1299, 1247, 992, and 944 cm^{-1}; UV (CH_3OH) 227 (ϵ 18900) and 299 nm (1590); ^{1}H-NMR (acetone-d_6, 400 MHz) δ 2.32 (3H, s), 2.66 (3H, s), 4.36 (1H, d, J = 15.8 Hz), 4.47 (1H, d, J = 15.8 Hz), 7.26 (1H, ddd, J = 7.3, 7.3, and 1.5 Hz), 7.37 (1H, ddd, J = 7.3, 7.3, and 1.1 Hz), 7.45 (1H, br d, J = 7.3 Hz), and 7.53 (1H, br d, J = 7.3 Hz); ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 2.39 (3H, s), 2.64 (3H, s), 4.37 (2H, d, J = 1.5 Hz), 7.25 (1H, ddd, J = 7.3, 7.3, and 1.0 Hz), 7.36 (1H, ddd, J = 7.3, 7.8, and 1.5 Hz), 7.48 (1H, br d, J = 7.3 Hz), and 7.51 (1H, br d, J = 7.8 Hz); EI-MS m/z (%) 248 (M^+, 1.4), 233 (0.6), 201 (1.4), 176 (15), 175 ([$C_{10}H_9NS$]$^+$, 100), 174 (13), 173 (11), 143 (10), 87 (15), 72 (19), and 44 (10).

Compound 18: $C_{12}H_{14}N_2O_2S$ (m/z 232.0697, $M^+ - H_2O$), gum; $[\alpha]^{18}_D$ = −9.21° (c 0.34, $CHCl_3$); IR ($CHCl_3$) 3342, 3320, 3008, 2926, 2852, 1681, 1617, 1488, 1468, 1323, and 965 cm^{-1}; UV (CH_3OH) 254 sh (ϵ 4850) and 391 nm (2330); ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 1.33 (3H, s), 2.31 (3H, s), 3.56 (2H, d, J = 5.9 Hz), 5.02 (1H, br s, D_2O exchangeable), 5.68 (1H, br s , t-like, D_2O not completely exchangeable), 6.84 (1H, dd, J = 7.8 and 7.3 Hz), 6.88 (1H, d, J = 8.3 Hz), 7.47 (1H, ddd, J = 8.3, 7.3, and 1.5 Hz), and 7.58 (1H, d, J = 7.8 Hz); ^{13}C-NMR ($CDCl_3$, DEPT, 100 MHz) δ 12.5 (q), 20.8 (q), 29.7 (s), 47.1 (t), 112.9 (d), 119.3 (d), 124.8 (d), 137.7 (d), and 160.8 (s); EI-MS m/z (%) 232 ($M^+ - H_2O$, 3), 202 ([M-SCH_3-H]$^+$, 14), 189 (13), 174 ([M-$COSCH_3$-H]$^+$, 24), 130 (21), and 47 (100); FD-MS m/z (%) 250 (M^+, 100).

Trapping Experiment of Indol-3-ylmethyl Isothiocyanate (10a). Twenty pieces of turnip root were homogenized in a Waring blender with 2.5 L of phosphate buffer (pH 7.0, 25 mmol dm^{-3}) containing 200 mL of 15% aqueous sodium methanethiolate for 5 min at room temperature. The homogenate was immediately poured into 1 L of ethyl acetate. The ethyl acetate layer was separated from the residue by centrifugation at 1000 rpm for 10 min. The organic phase was dried with sodium sulfate and evaporated *in vacuo*. The residue (1.075 g) was separated by silica gel column chromatography (benzene–ethyl acetate, 1:1), followed by

preparative TLC on silica gel to give phenethylbrassinin (**13**) (2 mg) and **1a** (2 mg). **1a** was identified by HPLC analysis and by direct comparison with authentic **1a**. A second compound was characterized as **13** on the basis of spectroscopic analysis.

13: $C_{10}H_{13}NS_2$ (*m/z* 211.0486, M^+), gum; IR ($CHCl_3$) 3376, 3062, 3026, 2960, 2922, 2854, 1493, 1453, 1383, 1336, 1307, 1260, 1217, 1095, 1030, 935, 908, 853, and 701 cm^{-1}; ^1H-NMR (90 MHz, $CDCl_3$) δ 2.62 (3H, s), 2.97 (2H, t, J = 6.8 Hz), 3.75 (1H, s, D_2O exchangeable), 4.00 (2H, m), and 7.29 (5H, m); EI-MS *m/z* (%) 211 (M^+, 2), 163 ([M-SCH_3-H]$^+$, 24), 105 ([$PhCH_2CH_2$]$^+$, 23), 91 ([$PhCH_2$]$^+$, 100), 77 (10), 72 (4), and 65 (12).

[*methyl*-$^2H_3$]Brassinin. To a mixture of 3-(aminomethyl)indole[16] (556 mg, 3.80 mmol, crude), dry triethylamine (580 μL, 4.16 mmol), and dry pyridine (2 mL) was added carbon disulfide (250 μl, 4.24 mmol) at 0 °C. The mixture was kept at 0 °C for 1 h, treated with [*methyl*-$^2H_3$]methyl iodide (99%, 260 μL, 4.01 mmol) at 0 °C, kept at room temperature for 18 h, and then poured into 1.5 M H_2SO_4 (50 mL). The mixture was extracted with ether (2 × 50 mL). The combined extract was washed with water (100 mL), saturated aqueous sodium hydrogen carbonate (100 mL), and water (100 mL), successively. The ethereal extract was dried over Na_2SO_4 and filtered, and the filtrate was concentrated *in vacuo*. The crystalline residue (436 mg) was recrystallized from dichloromethane to give [*methyl*-$^2H_3$]brassinin (311 mg, yield 34%). The ^1H-NMR spectrum of the synthetic [*methyl*-$^2H_3$]brassinin revealed a 99% incorporation of ^2H at the methyl group: ^1H-NMR ($CDCl_3$) δ 2.65 (0.01H, s), 5.07 (2H, d, J = 4 Hz), 7.00 (1H, br s), 7.18 (1H, ddd, J = 8, 8, and 1 Hz), 7.25 (1H, ddd, J = 8, 8, and 1 Hz overlapped with singlet 1H), 7.42 (1H, d, J = 8 Hz), 7.64 (1H, br d, J = 8 Hz), and 8.16 (1H, br s).

[*methyl*-$^2H_3$]Cyclobrassinin. To a solution of [*methyl*-$^2H_3$]brassinin (99%, 283 mg, 1.18 mmol) in dry dichloromethane (24 mL) was added pyridinium bromide perbromide (380 mg, 1.18 mmol). The mixture was kept at 0 °C for 20 min, basified with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU, 257 μL, 1.72 mmol) at 0 °C, and then kept at room temperature for 4 h. The reaction mixture was evaporated, and the residue was separated by chromatography on silica gel (25 g) with dichloromethane to give crystalline material (117 mg), which on recrystallization from dichloromethane–hexane gave [*methyl*-$^2H_3$]cyclobrassinin (69 mg) in 25% yield, indistinguishable from natural **2** except for the methyl signal. The ^1H-NMR spectrum of the synthetic [*methyl*-$^2H_3$]cyclobrassinin revealed a 99% incorporation of ^2H at the methyl group: ^1H-NMR ($CDCl_3$) δ 2.56 (0.01H, s), 5.09 (2H, d, J = 4 Hz), 7.14 (1H, ddd, J = 7, 7, and 2 Hz), 7.18 (1H, ddd, J = 7, 7, and 2 Hz), 7.33 (1H, dd J = 7 and 2 Hz), 7.49 (1H, dd, J = 7 and 2 Hz), and 7.91 (1H, br s).

DL-[*methyl*-$^2H_3$]Dioxibrassinin. 3-(Aminomethyl)dioxindole hydrochloride[17] (100 mg, 0.37 mmol) was dissolved in dry pyridine (500 μL) and cooled down with an ice bath. Triethylamine (100 μL, 0.74 mmol) and carbon disulfide (22 μL, 0.37 mmol) were added to the solution, and the mixture was stirred for an additional 4 h at room temperature. The reaction mixture was treated with [*methyl*-$^2H_3$]methyl iodide (99%, 260 μl, 4.01 mmol) at 0 °C, kept at room temperature for 18 h, then poured into 1 M HCl, and extracted with ethyl acetate three times. The combined extract was dried over $MgSO_4$, and the solvent was evaporated *in vacuo*. The residue was purified by column chromatography on silica gel (15 g) with methanol–dichloromethane (5:95) as eluent to give [*methyl*-$^2H_3$]-dioxibrassinin (94 mg, yield 78%). The ^1H-NMR spectrum of the synthetic [*methyl*-$^2H_3$]dioxibrassinin revealed a 99% incorporation of ^2H at the methyl group: ^1H-NMR (acetone-d_6) δ 2.70 (0.01H, s), 4.00 (1H, dd, J = 14.2 and 3.9 Hz), 4.53 (1H, dd, J = 14.2 and 5.9 Hz), 5.58 (1H, br s), 7.06 (1H, d, J = 7.6 Hz), 7.15 (1H, dd, J = 7.6 and 7.6 Hz), 7.40 (1H, dd, J = 7.6 and 7.6 Hz), 7.55 (1H, d, J = 7.6 Hz), 8.97 (1H, br s), and 9.63 (1H, br s).

Benzylbrassinin (14). Sodium methanethiolate (15% aqueous solution, 0.50 mL, 1.07 mmol) was added to a solution of benzyl isothiocyanate (113 mg, 0.76 mmol) in 5 mL of acetone and water (3:1), and the mixture was stirred for 10 min at room temperature. Acetone was removed *in vacuo*, and the mixture was poured into 20 mL of water. The mixture was extracted with ether (2 × 20 mL), and the combined ethereal extract was dried over Na_2SO_4. The solvent was evaporated *in vacuo* to leave almost pure **14** (136 mg, yield 91%): $C_9H_{11}NS_2$ (*m/z* 197.0311, M^+), colorless oil; IR ($CHCl_3$) 3328, 3058, 3024, 3000, 2914, 1497, 1452, 1424, 1377, 1333, 1303, 1235, 1089, 1065, 1028, 935, 820, and 734 cm^{-1}; UV (CH_3OH) 254 (ϵ 11800) and 270 nm (11000); ^1H-NMR ($CDCl_3$) δ 2.66 (3H, s), 4.91 (2H, br d, J = 5.4 Hz), 7.11 (1H, br s, D_2O exchangeable), and 7.34 (5H, s). The spectrum showed minor signals (1/3 the intensity of the major signals) due to a rotamer at δ 2.70 (s), 4.62 (d, J = 4.9 Hz), and 7.82 (br s, D_2O exchangeable); EI-MS *m/z* (%) 197 (M^+, 2), 149 ([Ph-CH_2-NCS]$^+$, 13), 91 (100), 65 (13), 48 (16), and 47 (18).

2-Methylindole-3-carboxaldehyde Oxime. Anhydrous sodium carbonate (1.10 g, 10.3 mmol) was added to a solution of hydroxylamine hydrochloride (700 mg, 10.1 mmol) in water (3 mL). 2-Methyl-3-formylindole (1.024 g, 6.43 mmol) in 20 mL of ethanol was then added to the hydroxylamine solution. The mixture was refluxed for 16 h and was allowed to cool to room temperature. After removal of the solvent under reduced pressure, the residue was redissolved in ethyl acetate (300 mL), washed with brine (2 × 300 mL), dried over Na_2SO_4, and concentrated *in vacuo*. Residual crystals were recrystallized from ethyl acetate to afford first crystals (99 mg) and second crystals (633 mg, total yield 75%): $C_{10}H_{10}N_2O$ (*m/z* 174.0787, M^+), mp 143–145 °C (AcOEt); IR ($CHCl_3$) 3370, 3272, 2970, 1612, 1574, 1552, 1455, 1413, 1309, 1246, 1154, 1140, 1102, 1028, 1010, 955, 906, 863, 830, 782, 746, and 664 cm^{-1}; UV (CH_3OH) 226 (ϵ 23300), 267 (16600), and 287 nm (13500); ^1H-NMR (acetone-d_6, 400 MHz) δ 2.64 (3H, s), 7.18 (2H, m), 7.43 (1H, d, J = 7.8 Hz), 8.12 (1H, d, J = 7.8 Hz), 8.47 (1H, s), 9.70 (1H, s), and 10.43 (1H, br s); EI-MS *m/z* (%) 174 (M^+, 52), 158 (23), 157 (77), 156 (100), 155 (99), 142 (10), 130 (36), 129 (10), 128 (16), 102 (10), 101 (15), 78 (23), 77 (18), 51 (14), 43 (15), and 40 (34).

2-Methylbrassinin (16). An aqueous solution (8 mL) of 1 M sodium hydroxide was added to a solution of the oxime (102 mg, 0.590 mmol) in methanol (3 mL), and the mixture was stirred at room temperature. Devarda's alloy (630 mg) was added to the solution with vigorous stirring, and the mixture was allowed to stand for 20 min. The mixture was cooled to room temperature with a water bath and filtered through a Büchner funnel. The filtrate was extracted with ether (3 × 30 mL). The combined extract was dried over Na_2SO_4 and evaporated *in vacuo*. Almost pure amine was obtained and used in the next reaction without further purification. To a mixture of 3-aminomethyl-2-methylindole (102 mg, 0.638 mmol, crude), dry triethylamine (0.1 mL), and dry pyridine (1 mL) was added carbon disulfide (100 μL, 1.70 mmol) at 0 °C, and the mixture was kept at this temperature for 1 h. The reaction mixture was then treated with methyl iodide (100 μL, 1.61 mmol) at 0 °C, kept at room temperature for 18 h, poured into 0.25 M H_2SO_4 (50 mL), and extracted with ether (3 × 30 mL). The combined ether extract was washed with 0.25 M H_2SO_4 (100 mL), water (100 mL), saturated aqueous sodium hydrogen carbonate (2 × 100 mL), and water (100 mL), successively. The ethereal extract was dried over Na_2SO_4 and evaporated *in vacuo*. The resulting crystals (120 mg) were purified by column chromatography on silica gel (10 g) with benzene to give **16** (106 mg, 72% from oxime): $C_{12}H_{14}N_2S_2$ (*m/z* 143.0759, $C_{10}H_9N$), mp 119–121 °C (benzene); IR ($CHCl_3$) 3462, 3368, 3002, 2918, 1460, 1377, 1298, 1247, 1222, 1078, 1008, 956, and 929 cm^{-1}; UV (CH_3OH) 222 (ϵ 36100) and 271 nm (15400); ^1H-NMR ($CDCl_3$, 400 MHz) δ 2.44 (3H, s), 2.64 (3H, s), 4.98 (2H, d, J = 4.4 Hz), 6.91 (1H, br s), 7.12 (1H, dd, J = 7.8 and 6.8 Hz), 7.17 (1H, dd, J = 7.8 and 6.8 Hz), 7.30 (1H, d, J = 7.8 Hz), 7.50 (1H, d, J = 7.8 Hz), and 7.97 (1H, br s). The spectrum showed minor signals (1/5 the intensity of the major signals) due to a rotamer at δ 2.17 (s), 2.83 (s), 4.70 (d, J = 4.5 Hz), and 7.81 (br s); EI-MS *m/z* (%) 144 (14), 143 (100), 142 (17), 128 (10), 116 (11), 115 (18), 107 (12), 102 (42), 76 (19), 75 (12), 60 (15), 59 (54), 51 (12), 50 (11), 48 (44), 47 (52), 45 (31), and 44 (11); FD-MS *m/z* (%) 250 (M^+, 100).

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Supplementary Material Available: Text and diagrams describing the structures of compounds **22** and **23**, text describing the experimental methods of characterizing **22** and **23**, and a table of the radioactivity data of labeled compounds (9 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.